Title:

Use of Nylon as an In Vitro Scaffold for 3D Study of Neural Cells

Abstract:

The requirement for improved modelling of cells in culture for study of cell-to-cell interactions has led to an increased focus on three-dimensional (3D) in vitro models. In this study, neural cells and Schwann cells have been cultured on nylon mesh substrates and have been shown to remain viable on the biocompatible fibres. Namely, the neural cell line NG108-15 cells and rat sciatic nerve Schwann cells were cultured as separate monocultures and together in a co-culture on the nylon mesh. This has allowed for cell-to-cell interactions between the two cell types to be monitored and recording of cell proliferation on the mesh fibres over time. Electrophysiological recordings have been used to validate NG108-15 cell differentiation when cultured on the nylon mesh substrate. Action potentials were recorded and were comparable to results from two-dimensional (2D) culture. A variety of assays have been used, demonstrating the use of nylon as an appropriate substrate for neural cell in vitro studies, and nylon can easily be imaged using inverted microscopy. These studies inform on the suitability of the nylon 3D culture presented for modelling neural cell behaviour.

Keywords:

Three-dimensional culture, nylon, NG108-15, electrophysiology.

Introduction:

In Neuroscience, two-dimensional monolayer cell culture has allowed for important cellular mechanisms and modelling studies to be performed, such as the study of homeostatic plasticity[1]. Typically, two-dimensional (2D) cell cultures are performed on glass petri dishes or polystyrene well plates[2,3]. Such techniques allow for analysis of cell behaviour in vitro, which can then be correlated to in vivo physiology thus advancing the field.. However, two-dimensional cultures are often criticised for imposing limitations on cell differentiation, proliferation and synapse formation. The three-dimensional (3D) extracellular matrix that cells lay down is considered vital for the population to be able to maintain homeostasis[4]. Consequently the provision of depth within a 3D culture may allow for more naturalistic axonal sprouting – which in turn permits increased connectivity that may be more physiologically relevant[5].

The increase in the volume of the cell culture can allow for more diverse connections to be established. Physical cues have been shown to affect the orientation of axon extension in axon guidance studies[6,7]. Therefore provisions of a larger culture volume within 3D cultures can allow for increased axon growth and monitoring of axon development and regeneration[8,9,10]. Neurons specifically are dissimilar to other classes of cell in that they extend their processes over remarkable distances, in the 100 µm range in vitro[2]. It has been

reported that the three-dimensional environment allows for more reliable study of microenvironments within the cell itself[11,12]. This agrees with the hypothesis that an increase in cell culture volume increases the number of relevant interactions between cells for modelling of cell physiology. This data can be specifically useful in informing strategies for regenerating tissues where cellular interactions with biomaterials are analysed.

Traditionally, 3D in vitro study has involved use of hydrogels containing extracellular matrix proteins, for example collagen hydrogels[13]. Although 3D cell cultures have effectively been published using cellular encapsulation methodologies, cells are forced to interact with these materials typically made from bovine collagen type 1, poly(ethylene glycol (PEG) or Poly(acrylamide) gels[14]. The use of non-hydrogel scaffolds for 3D cell culture may provide a means to study alternative cues to cells in vitro, specifically by use of 3D solid-phase substrates rather than 3D gel-phase substrates[15]. This may help to mitigate the effect of hydrogel substrate stiffness in stem cell differentiation studies[16]. Fluctuation in viscoelastic stiffness (kPa) of hydrogels can affect cell physiology as reported; affecting cellular growth, proliferation and motility which can confound 3D culture data[17,18]. It is important for 3D studies to not be influenced by fluctuation in mechanical stability which can affect cell attachment as these studies include biomaterials intended for use in tissue engineering. It has been shown that minute details in cell surface substrate geometry such as inclusion of printed cell adhesion motifs can allow for control in neuritogenesis, which can be more

easily controlled on solid-phase 3D culture substrates and translate to an appropriate peripheral nerve guidance strategy for example[19].

Recently, attempts to mimic cell-to-cell interactions in the field of peripheral nerve regeneration have resulted in fabrication of neurospheroids by encapsulating neural cell line NG108-15 hybridoma cells with Schwann cells[20]. The research reports extension of axon-like processes within a collagen gel, and also highlights the disadvantage of unsuccessful quantification of the length of the outgrowth which was intended as a dependent variable. As the concentration of the hydrogel increases key variables for reliable image acquisition such as signal to noise ratio, spatial resolution and a large field of view can be compromised, especially if the components of the hydrogel autofluoresce [21,22]. Furthermore, batch-to-batch variation in gel constituents, can limit reproducibility between experiments making 3D hydrogel culture difficult for use in drug screening[23]. A key advantage to the spheroid culture is the ability to record cell-to-cell interactions in 3D space, from which data can be used to replace and reduce use of animal tissue[9]**.** However, the spheroidal structure also results in randomly structured cultures which can be overcome using a rigid, carefully designed scaffold which can allow for self-organisation of co-cultured cells[24]. Nanofibers have been fabricated for use in research on nerve regeneration in vivo, made of carbon nanotube which may be functionalized to be electrically conductive or electrospun to be highly aligned, for example with use of poly(lactide-co-glycolide) polymer[25]. The fibres have

been shown to promote functional recovery of nerve crush injury in vivo and evaluated in a further study which shows the importance of three-dimensional in vitro cultures as a tool for predicting success of three-dimensional applications in vivo[26].

The use of 3D cell culture is increasing, predominately due to need for research to reliably monitor and follow cell-to-cell interactions to predict mechanisms relevant to pathology or regeneration. Here, we report use of nylon as an inexpensive culture platform, as highlighted previously where nylon meshes were shown to be cell-adherent. Nylon is available in a mesh form, with standardised lattice spacing available, allowing for control of cell proliferation on the substrate in a topological manner. Nylon shows excellent biocompatibility, cell adherent properties and is mechanically durable for handling and transfer of cultured cells between well plates, unlike many 3D hydrogel systems[27]. 3D cultures consisting of soft hydrogels can be damaged during post-culture processing without careful attention such as during staining protocols for imaging.

The objectives of this study was to assess the biocompatibility of nylon fibre mesh, viability of cultures and cell differentiation of NG108-15 cells and Schwann cells to evaluate the use of nylon cultures in researching cells pertinent to peripheral nerve regeneration research and cell modelling.

Methods and Materials:

Cell Culture:

NG108-15 cells (ATTC, trading as LGC Standards, UK) were cultured as described previously[28]. NG108-15 cells were proliferated in Growth Media (GM), which was made using Dulbecco's Minimal Essential Media (DMEM) (Sigma-Aldrich) with 10% fetal calf serum (FCS) (Sigma-Aldrich), 100µM Hypoxanthine, 0.4 µM Aminopterin and 16µM Thymidine (HAT Supplement, Sigma-Aldrich), Pennicillin/ Streptomycin (P/S) and Glutamax (Thermo Fisher Scientific, UK). After 24 hours, cells were differentiated in differentiation media, DM, which was made in DMEM with 1% Fetal Calf Serum, 0.25mM 3-Isobutyl-1-mehtylxanthine (Sigma-Aldrich), 3µM Prostaglandin E1 (Sigma-Aldrich), P/S and Glutamax. After 24 hours in DM, cells were maintained in plating media, PM, was made using 1% FCS, DMEM, P/S and Glutamax until the experimental endpoint.

Schwann cells (Sigma) were cultured in a defined, proprietary Schwann Cell media (ScienCell Research Laboratories) containing 10% FCS (ScienCell Research Laboratories), Schwann cell growth supplement (SCGS) (ScienCell Research Laboratories) and Pennicilin/Streptomycin.

Schwann cell media was used for co-culture of NG108-15 cell and Schwann cell samples. This is because HAT supplement is designed for hybridoma cell culture only, and non-hybridoma cells such as the Schwann cells will not proliferate[29].

Abbreviations for cell cultures are as follows; NG- NG108-15 cell line culture, SC – Schwann cell culture, NGSC – NG108-15 cell and Schwann cell co-culture.

Plating Protocol:

Cells were cultured on static, uncoated, non-treated nylon mesh in polystyrene culture wells and polystyrene cell culture well surfaces for the control condition. After initial attachment for a 24-hour period, the nylon mesh was carefully withdrawn from the culture well containing growth medium and placed into a new well. This is to ensure that cells that had not adhered to the nylon did not affect the results of the assays carried out. Cells were proliferated and differentiated on the nylon in the new wells and maintained until the desired time point at either day 1, day 3, or day 7 dependent on the assay. The nylon mesh consists of uncoated, non-treated nylon fibres which are 27 µm in diameter. The mesh structure includes a maintained aperture of 80µm, which is the distance between two parallel fibres within the mesh. The gross structure can be observed in Fig. 1.

[FIGURE 1]

Live/ Dead Analysis:

Live/Dead cell viability assay (Sigma-Aldrich) was carried out using a live/dead imaging solution containing both Calcein AM (4µl) and Ethidium Homodimer-1 (10µl) diluted into 10ml of PBS. 24 well plates containing cultures were aspirated to remove the media and a minimal

volume of 200µl of the live/dead imaging solution containing Calcein AM and Ethidum Homodimer-1 was applied per well and incubated for 30 minutes at 37^{o}C, 5% CO_2. Images were obtained by epifluorescence widefield microscopy.

Alamar Blue Assay:

The Alamar blue assay measures the ability of cell mitochondria to reduce resazurin to resorufin which is fluorescent. Alamar Blue solution was prepared by dissolving resazurin (Sigma-Aldrich) in PBS at a 1% w/v concentration. Alamar blue medium was produced by mixing Alamar Blue solution with warmed PBS in a 1:10 ratio to make the working concentration of Alamar Blue solution. The solution was applied to wells containing cells (ctrl) and wells with cells cultured on nylon, and allowed to incubate at 37^{o}C, 5% CO_2 for 4 hours. Following incubation, 100µl aliquots from the wells were removed and loaded into the wells of a 96-well plate. Using a microplate reader, absorbance was calculated at 570/600nm and recorded. The experiment was performed in triplicate, **with each replicate using separate cell passage sources**.

Patch clamping:

An electrophysiological study was carried out using a previously published protocol. Cells were plated onto 35-mm petri dishes (Nunclon) for the negative control condition, and cultured on uncoated, non-treated nylon mesh for the test group (named ‘Mesh’ in Fig. 6) as

for live/ dead analysis and Alamar blue assay. Cells were differentiated as described above on the well plate and the nylon mesh, and used at day 4-7 for recording. Non-differentiated cells were recorded before differentiation medium was applied, between day 1 and day 2, and used as a positive control. For recording experiments, the medium was replaced with a superfusate external solution containing 120mM NaCl, 3mM KCL, 1.2mM $MgCl_2$, 22.6mM $NaHCO_3$, 11.1mM glucose, 5mM HEPES (N-2-hydroxyethyl piperazine N'-2-ethane sulfonic acid), 2.5mM $CaCl_2$ adjusted to pH 7.36. Cells were patch clamped in the whole-cell mode, using a discontinuous current-clamp. Electrodes (3-5MΩ) were filled with 90mM $C_2H_3KO_2$, 20mM KCl, 40mM HEPES, 3mM EGTA ([ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid), 3mM $MgCl_2$, 1mM $CaCl_2$ adjusted to pH 7.4 with KOH. Action potentials were evoked by current jumps, ranging from 1-5.5 nA, generated by PC-based software (pCLAMP, Axon Instruments) via a current clamp amplifier (Axoclamp2, Axon Instruments). Voltage was digitally recorded on computer. The membrane potential was held at -50mV and intermittently the current was clamped to a series of test currents in 0.5nA intervals over a range of 1-5.5nA, for 1 second every 15 seconds. For the Current/ Voltage (I/V) curve, Maximal Voltage (Vmax) was recorded and plotted against current.

Image analysis:

Well plates were imaged using inverted epifluorescent microscopy (Olympus) and images taken using PC based software (CellSens). The air objective used was 20X and at least three

images were taken randomly from the well and repeated for 3 wells in 3 independent experiments. Fluorescent images were analysed using Image J software, where total area of image fluorescence was calculated.

Statistics:

Measurements taken from Image J were tabulated and One Way ANOVA was used to compare means with post-hoc Tukey test, which were performed using statistical software (SPSS). For the live/dead analysis, 4 fields of view were taken for each sample group from independent experiments in triplicate (n=12). For the Alamar Blue assay samples of each group were analysed in 3 independent experiments with 4 absorbance readings taken per sample (n=36). Standard error of the mean (SEM) is displayed in Fig. 4, 5 and 6.

Results:

Live/ Dead Analysis

The NG108-15 cells and Schwann cells were cultured on the tissue culture plastic, and showed a 53.0% and 24.3% increase in fluorescent area respectively from DIV3 to DIV7. Cells show two distinct phenotypes, which are well characterized morphologically. NG108-15 cells show a neural cell phenotype when differentiated and grow considerably long neurites from the cell body; morphology can be seen in Fig. 2A. Schwann cells are spindle-like with either a

bipolar or multipolar morphology and are shown to spread out on the substrate as seen in Fig. 2B.

[FIGURE 2]

Cells show excellent adherence onto the nylon, at both day 3 and day 7. There is a clear proliferation of both cell types (NG108-15 cells and Schwann cells) on the nylon itself as shown by data calculating total area of image fluorescence. Mean fluorescent area of NG108-15 cells and Schwann cell cultured on nylon increased by 21.8% and 36.9% respectively from DIV3 to DIV7. This shows a clear proliferation of both cell types on the nylon substrate, which can be clearly seen in Fig. 3 where both cell types are shown adhered to the nylon fibres exclusively.

[FIGURE 3]

The percentage of live cells was calculated by the ratio of live cells (Calcein AM positive cells) to dead cells (Ethidium homodimer-1 positive cells) in all experimental conditions. When cells were cultured on the tissue culture well surface or nylon mesh, the percentage of live cells was quantified. This was achieved by measuring the number of Calcein AM positive cells and calculating the percentage of these cells over the total number of live and dead cells. The mean percentage of cells stained with Calcein AM on nylon mesh was calculated to be 98.86% for the NG culture, 99.30% for the SC culture, and 99.28% for the

NGSC co-culture at DIV7 as in Fig. 4. Comparably, the mean percentage of live cells cultured on the control polystyrene culture well surface was calculated to be 99.30% for the NG culture, 98.38% for the SC culture, and 99.72 for the NGSC co-culture at DIV7.

Regarding mean area of live cells per image, when NG108-15 cells and Schwann cells were co-cultured on nylon the mean area of cells was significantly larger than the NG and SC monocultures on nylon at day 7. The mean area for the NGSC co-culture was 174,859.86 $pixels^2$ +/- 22,784.04 (SEM) which was larger compared to the NG monoculture (86,688.73 $pixels^2$ +/- 11,907.39 (SEM)) and the Schwann cells (105,888.30 $pixels^2$ +/- 21,547.21 (SEM)) both in single cell cultures on nylon mesh. When a one-way ANOVA with post-hoc Tukey test was performed, the area of live cells of NG108-15 cells on nylon, Schwann cells on nylon and mixed NG108-15 and Schwann cells on nylon were compared at day 7. The area of live cells in the mixed co-culture condition was significantly increased ($p<0.05$) compared to the single cell cultures. The calculated p-values show statistically significant differences between mean live cell area values where $p=0.0035$ for NG108-15 cells on nylon vs. co-culture on nylon and $p=0.0294$ for Schwann cells on nylon vs. co-culture on nylon. Therefore, significant increases in live cell area in the co-culture compared to the monocultures on nylon were calculated (as seen in Fig. 4). Furthermore, at day 7 the NGSC co-culture shows no statistical difference between the mean area of live cells on the nylon mesh when

compared to cells co-cultured in the control condition (p=0.856). This result compares well to the proliferation calculated of the co-culture on the nylon mesh.

[FIGURE 4]

Alamar Blue Assay

[FIGURE 5]

The Alamar Blue assay was carried out to validate the proliferation of NG108-15 cells and Schwann cells on nylon at time points of day 1, day 3 and day 7. The cultures analysed were either monocultures (containing NG108-15 cells or Schwann cells) or co-cultures of NG108-15 cells and Schwann cells on the control culture well surface or on the nylon mesh. As seen in Fig. 4, on day 1 mean absorbance for the cell cultures on nylon ('Nylon NG', 'Nylon SC', 'Nylon NGSC') were comparable in absorbance readings (0.052, 0.060 and 0.050 respectively) and had on average 33.58% of the total cells adhered to the nylon at day 1. Over the course of 7 days, absorbance values indicating adherent cells on nylon all increased as they did on the control culture well surface. Adherent NG108-15 cells on nylon increased in absorbance to 0.2207 +/- 0.0118 (SEM) by day 7, with a percentage increase of 328% from day 1 to day 7. Similarly, in the NGSC co-culture, the absorbance increased by 308% from day 1 to day 7. The proliferation of Schwann cells on nylon maintained relatively constant over 7 days, absorbance values of 0.0598 at day 1 and 0.0574 at day 7. An ANOVA

with post hoc Tukey test was carried out for absorbance data at day 7. NG108-15 cells on nylon and the NG108-15 cells/ Schwann cell co-culture on nylon mean absorbance values were significantly higher in comparison to Schwann cells cultured on nylon ($p=0.0010$).

Electrophysiological Recordings of NG108-15 cells cultured on nylon

Electrophysiological recordings were taken from NG108-15 cells, to validate differentiation of the cells cultured on the nylon substrate, by conservation of the action potential amplitude. As Fig. 6 shows, action potentials were elicited by current injection and recorded in the whole cell configuration from differentiated NG108-15 cells. Fig. 6C shows the current/ voltage (I/V) curve for differentiated NG108-15 cells cultured on the control cell culture well surface, differentiated NG108-15 cells cultured on nylon mesh, and undifferentiated NG108-15 cells cultured on the control culture well surface. The smallest current injection of **1nA caused action potential events to be recorded in some of the differentiated NG108-15 cells cultured** on both nylon and culture well surface. As more current was injected in a stepwise manner, the differentiated NG108-15 cells responded by increasing the maximal voltage (Vmax) recorded when cultured both on tissue culture plastic and nylon which was plotted on the I/V curve. Student's t-test was performed to test statistically significant differences between Vmax data from differentiated NG108-15 cells cultured on the culture well surface and differentiated NG108-15 cells on nylon. No significant difference was calculated between mean Vmax values at each amplitude injected

in differentiated cells, as seen in Fig. 6C. For example, the mean Vmax recorded for differentiated NG108-15 cells on nylon was 146.67mV and the mean Vmax for differentiated NG108-15 cells on the culture well surface was 108.26mV. The p-value calculated when comparing cells from the nylon and control culture was greater than 0.05 so no significance was observed at **t**he highest current injection of 5.5nA where p=0.1056. However when the t-test was performed to compare differentiated cells cultured on nylon to undifferentiated cells cultured on the well surface, there was a highly statistically significant difference between mean Vmax recorded at 5.5nA (p=0.00001). This indicates that the NG108-15 cells were able to mature on the nylon without losing their electrophysiological capability and similar I/V curves were produced, in the same confidence interval, regardless of culture surface.

Discussion:

Live/Dead Analysis

NG108-15 cells cultured on nylon showed equivalent long-term viability over 7 days to that of the control sample (culture well surface), when analysed by live/dead staining with equal numbers of cells in both groups. Similarly, the adult rat Sciatic nerve derived Schwann cells were also cultured on the nylon mesh and have shown excellent viability as indicated by calculation of the percentage of live cells. When the cells were cultured together in a 1:1 co-

culture with equal numbers of cells, the high percentage of cell viability was conserved (>90%).

As cells were also analysed by image analysis there was a proliferative effect when the NG108-15 cells and Schwann cells were cultured together (as seen in Fig. 2 and 3). Schwann cells have previously been reported to increase neurite length through cell signalling in conditioned media[30,31]. Monitoring co-culture effects is vital to understanding peripheral nerve regeneration in vivo, and can be analysed using nylon mesh in 3D culture as the mesh is clearly unobtrusive to cell-to-cell interactions[32]. The mesh system allows simple assays such as the live/dead cell viability assay to provide useful data for informing 3D repair strategies involving cultured cells[33].

The technique did not require a complicated imaging protocol and can be monitored using inverted epifluorescence widefield microscopy. Typically, due to the working distance introduced when culturing in 3D, confocal microscopy can accommodate the longer working distance and therefore help to increase resolution of 3D cultures[34,35]. Due to laser intensity and the requirement for fixation of samples; samples do not survive well after 1hr of confocal microscopy due to photobleaching. Live cell assays however, such as the Live/Dead assay conducted in this study, are an effective alternative[36]. This highlights the advantageous use of nylon mesh in three-dimensional live cell imaging assay. Furthermore, when cells were cultured in single cell type cultures or co-culture, it is clear that they are

able to proliferate on the nylon surface and were often observed to spread along the longitudinal axis of the nylon fibre. This data can be useful to biomaterials research, where fibre diameter and other structural details have been shown to affect Schwann cell morphology, for translational work in vivo[37,38].

Over time, the number of cells also increased between day 3 and day 7, demonstrating proliferation of the cells on the mesh substrate. The rate of proliferation has been shown to be conserved between the 2D and 3D culture surface substrates tested. The data presented proves proliferation data that can be correlated to previous investigations in 2D which may be used as control data for 3D culture investigations. It was an interesting observation that in the NGSC co-culture, the mean area of live cells within the field of view on nylon mesh when compared to NGSC co-culture on culture well surface was not statistically significant. However, the culture still provided a platform in which cells can interact in the 3D space, shown by the growth of cells over the aperture of the nylon mesh in Fig. 2E. This indicates a clear use of the nylon mesh substrate as a 3D tissue culture platform for imaging studies and live cell assays. In this study, comparable numbers of cells and appropriate fluorescent cell signal was obtained using widefield microscopy on the nylon meshes. The culture could be grown in layers to provide comparable numbers of cells to tissue slices and reduce the need for animal use. Conventionally accepted numbers of images for statistical analysis may remain equal between traditional 2D and the novel 3D cell culture presented providing a

useful alternative to 2.5D hydrogel studies which experience increased signal to noise ratio in widefield imaging[39]. The nylon mesh, or other structures, may be optically sectioned for analysis whilst also providing a 3D environment for cells to proliferate and communicate. The results shown here indicate that cells can be cultured on the mesh substrate and then transferred to a novel environment when the appropriate cell density is achieved[40]. For example, two meshes cultured with separate cell types can be introduced to the same well and media environment. This method could provide an alternative to feeder cultures used to study paracrine effects which rely on a 2D monoculture of Schwann cell feeder cells in peripheral nerve models which may not be as indicative of physiology as a 3D feeder cell system[41].

Alamar Blue Assay

The Alamar blue assay provided an alternative insight into the fraction of cells adhering to nylon after 24 hours. A third of the cells plated adhered to the mesh substrate, indicating that a dense population of adherent cells to the nylon can be achieved. The survival of these cells over the time of experiments could be monitored reliably. Cells show proliferation between 1, 3 and 7 days. Corresponding to the live/ dead data, cells increased in number as time in culture increased when in co-culture. Also, the numbers of cells cultured on the nylon mesh increased considerably over the culture time. This shows great potential in using nylon as an experimental three-dimensional substrate between 3 and 7

days in vitro, allowing proliferation time for cells to become more confluent in this time period. The use of this assay in this experiment confirms the use of Alamar blue assay, and similar colorimetric assays, to be conducted on 3D cultures using nylon. Aliquots may be taken at set time points and removed for microplate reading without disrupting cells adhering to the nylon or terminating the use of the sample. Furthermore, it has been indicated that hydrogels can impede the take-up of resazurin dye by cells affecting results. In this study, the use of the nylon 3D culture would avoid anomalies in absorbance readings without affecting cell 3D morphology, metabolism and adherence[42].

Schwann cells cultured on nylon did not produce as high readings of absorbance when compared to the NG108-15 cells or co-cultured NG108-15 and Schwann cells on nylon, nor when compared to the control Schwann cell culture. This is an interesting result as it may suggest that either Schwann cells do not proliferate well on nylon or that the considerably high reading of the NGSC co-culture was mostly contributed to by the NG108-15 cells present in the co-culture. It is also possible that Schwann cell mitochondria did not reduce the Alamar Blue resazurin as efficiently at the concentration used in this study, and this could be revisited or similar assays could optimise this aspect[43]. This result does not directly compare to the large areas of fluorescent Schwann cells witnessed in live cell imaging which showcased the adherence of these cells in the 3D culture. It could be the case that the Schwann cells are hindered from proliferating at a comparable rate to the control condition

when there are fewer cells present on the nylon mesh due to competing with NG108-15 cells for space to adhere to the mesh[44]. The result highlights that 3D cell culture on nylon requires distinct incubation periods for cells to become confluent on the nylon, which is most likely variable between cell types. Although the difference in readings may be a consequence of cell-to-cell interactions between the two cell types. The Schwann cells reach confluence at a slower rate when compared to NG108-15 cells. Assays such as the Alamar blue can be used to determine the time point at which three-dimensional cell culture on nylon is confluent and fit for use in experimental data acquisition. Due to not using immunocytochemistry It is difficult to determine the ratio of Schwann cells to NG108-15 cells in the co-culture, however they were initially seeded at a 1:1 ratio. The actual density of both cell types on the 3D mesh can be easily quantified using immunocytochemistry as indicated by the excellent signal to noise ratio in the live/dead analysis (see Fig. 2). The current 3D nylon platform allows for 3D cultures to be analysed and can be more useful in determining the correct ratio of Schwann cells to be included in a synthetic nerve graft for instance where information on cell numbers for encapsulation in a peripheral nerve guide is lacking[45]. Understanding the interdependence of neural and Schwann cells can inform on effective peripheral nerve grafting which has been demonstrated in this 3D co-culture with relevant cells[26]. The results clearly show that Schwann cells proliferate on the nylon mesh substrates in a time dependent fashion (see Fig. 5). This 3D culture could be indispensable in

experiments comparing suitable candidate cells for inclusion in a peripheral nerve graft which is an ongoing research question in the field, as the cells can be reliably cultured on the mesh and their effects on neural cell growth can be measured[46].

Electrophysiological recording of NG108-15 cells on nylon

Electrophysiological experiments have been used to validate the use of nylon mesh as a three-dimensional substrate for cell culture. NG108-15 cells have been shown to successfully adhere and differentiate when cultured on nylon. If nylon mesh is to be used in 3D neural cell experiments, the nylon mesh should not affect cell genotype or physiology. In this experiment, whole cell patch clamping of the cells using microelectrodes has allowed for analysis of action potential recordings. Even in the 3D configuration, action potentials can be recorded reliably from the nylon mesh itself without more difficulty than in the 2D culture. This result showcases the use of whole cell recordings on a 3D scaffold, which advances techniques in electrophysiology and provides an alternative to neural slice recordings and 3D reconstruction of cells by tissue staining[47,48]. Further, nylon mesh may be manipulated for other electrophysiological recordings such as field recording or calcium transient imaging as the mesh was shown not to affect action potential generation over a range of current injection amplitudes[49]. The maximal voltage recorded increased in a similar trend independent of culture substrate (nylon or control culture well surface). The key advantage of recording electrophysiological data from the nylon mesh is that functional

circuits may be selectively cultured as there is a growing interest in 3D functional physiology[50]. Recordings can be compared between nylon meshes in various studies relevant to peripheral nerve regeneration in this instance through paracrine signalling or pharmacological treatment manipulations[51]. Whilst still permitting three-dimensional cell-to-cell interactions, the current nylon mesh can allow for neuronal electrophysiology to be manipulated and analysed. Previously, circuits have been determined using optogenetics, and the same toolkit can be applied to discovering connectivity within the nylon substrate culture and uncovering important mechanisms of neural connectivity[52].

Conclusion

Nylon mesh has been used in these studies for three-dimensional cell culture of NG108-15 and Schwann cells as monocultures or jointly as a NG108-15 and Schwann cell co-culture. The Nylon mesh shows excellent biocompatibility assessed by live dead image analysis and also Alamar blue assay over 7 days in vitro. Nylon mesh has also been successfully imaged using inverted epifluorescence microscopy, which is commonly accessible in laboratories. Electrophysiological experiments were conducted on NG108-15 cells cultured on nylon mesh and action potentials have successfully been recorded to show electrophysiological function. Nylon mesh stands as an excellent candidate for three-dimensional cell culture as both cell types studied show correct cell morphology on the nylon fibres as compared to cells cultured on the culture well substrate, and furthermore the NG108-15 cells

demonstrated ability to differentiate on nylon. The nylon mesh format also allows for many cellular based assays to be performed in the same way as traditional monolayer cultures as the culture methodology is versatile and simple. This is specifically true for assays in which data is derived from cell culture medium aliquots such as the case for the Alamar Blue assay. Future work in this field would look to investigating cell-to-cell interactions on the nylon mesh in a specified three-dimensional array, which can inform emergent techniques in tissue engineering such as single cell bio-printing and three-dimensional neural network culture. The next stage of the current study would be to assess differentiation of NG108-15 cells on nylon in response to increasing ratios of Schwann cells to NG108-15 cells in co-culture to determine an in vitro model for peripheral nerve. Modelling cell function within a three-dimensional environment allows research to investigate more closely the dependency between cellular populations to carry out regenerative functions in peripheral nerve regeneration for instance, which can then be translated into in vivo models.

Declaration of Interest:

Conflicts of interest: none.

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Figure Legends:

Figure 1:

Figure 1: Nylon Mesh photomicrograph showing the gross structure. The micromesh patterning allows for a large surface area on which the cells used in the studies may proliferate. Scale bar - 50µm.

Figure 2:

Figure 2: Fluorescently labelled cells cultured on tissue culture plastic as a control substrate. NG108-15 neuronal cells (A) extend long processes. Schwann cells (B) are fusiform like cells. Scale bar – 50µm.

Figure 3:

Figure 3: Live/ Dead imaging. Calcein A and C) and ethidium homodimer (B and D) staining of mature NG108-15 cells and Schwann Cells on nylon at Day 3 respectively. At Day 7 the co-culture showed the greatest increase in number of cells (E). Scale bar 50µm.

Figure 4:

Figure 4: Bar charts showing percentage live cells, NG108-15, Schwann and mixed NG108-15 and Schwann cell culture on tissue culture plastic and nylon mesh substrate at Day 3 and Day 7 (top left and top right at DIV3 and DIV7 respectively). Bar chart showing mean area of fluorescent live cells for each condition (bottom) *$p<0.05$.

Figure 5:

Figure 5: Alamar blue data from NG108-15 cells (NG), Schwann cells (SC) and mixed co-culture of both cell types (NGSC) cultured on tissue culture plastic or nylon at Day 1, Day 3 and Day 7. **$p<0.01$.

Figure 6:

Figure 6: A single NG108-15 cell cultured on nylon mesh and patched by electrode during electrophysiological recordings (A). Typical trace of action potentials peaking at maximal voltage of 180mV showing 10 subsequent traces for each corresponding current injections, from 1-5.5nA with 0.5nA intervals (B). Current x voltage curve (I/V curve) showing mean maximal voltage for each current injection amplitude tested, with recordings taken from cells cultured on tissue culture polystyrene (‘Ctrl’ line), differentiated NG108-15 cells cultured on nylon mesh (‘Mesh’ line) and undifferentiated cells cultured on tissue culture polystyrene (‘Undiff’ line) (C).